



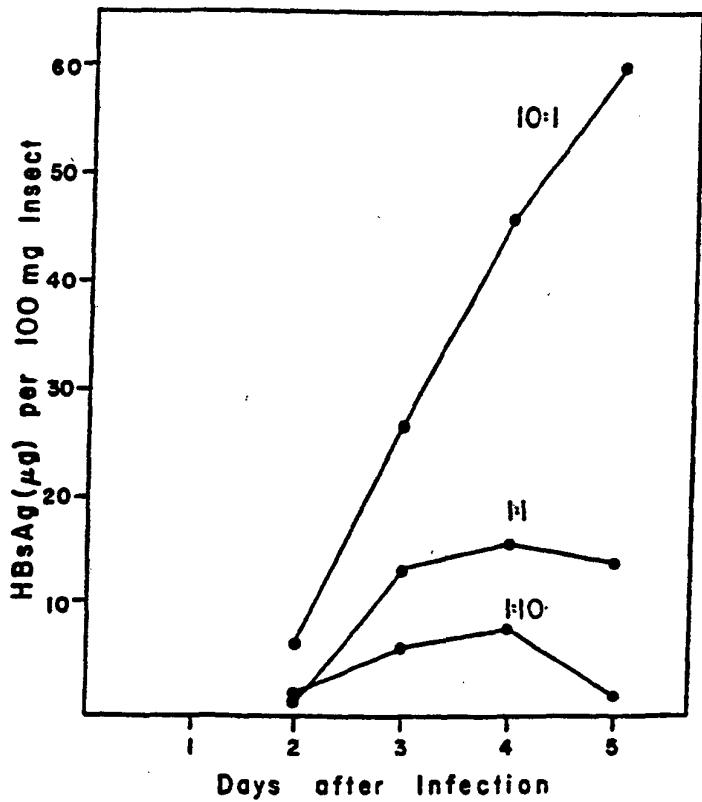
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12P 21/02, C12N 15/86, 15/36	A1	(11) International Publication Number: WO 90/01556 (43) International Publication Date: 22 February 1990 (22.02.90)
<p>(21) International Application Number: PCT/US89/03542 (22) International Filing Date: 4 August 1989 (04.08.89) (30) Priority data: 229,157 5 August 1988 (05.08.88) US</p> <p>(71) Applicant: MOUNT SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK [US/ US]; One Gustav L. Levy Place, New York, NY 10029 (US).</p> <p>(72) Inventors: ACS, George ; 78 Hilltop Drive, Manhasset, NY 11030 (US). PRICE, Peter ; 148 E. 38th Street, New York, NY 10016 (US).</p> <p>(74) Agents: MURNANE, John, D. et al.; Brumbaugh, Graves, Donohue & Raymond, 30 Rockefeller Plaza, New York, NY 10112 (US).</p>		<p>(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).</p> <p>Published <i>With international search report.</i></p>

(54) Title: IN VIVO INFECTION OF LIVE INSECTS WITH A RECOMBINANT BACULOVIRUS

(57) Abstract

This invention is a method for effecting high levels of expression of a foreign gene in a live insect. It involves creation of a novel recombinant baculovirus (AcNPV) which contains a foreign gene substituted for the polyhedrin gene but which still bears a polyhedrin nucleocapsid. The recombinant virus is ingested by the insect and causes an infection which efficiently produces large quantities of authentic foreign protein. The protein can be purified from insect larvae. As one example of the application of this method, hepatitis B virus surface antigen (HBsAg) protein was produced by live insect larvae and was shown to be both antigenic (as determined by a configuration-dependent radio-immunoassay) as well as immunogenic.



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Description

In vivo infection of live insects with a recombinant baculovirus.

5 Background of the Invention

This invention relates to a method which allows for the efficient, high level expression in live insects of a foreign gene in a recombinant baculovirus.

10 Related Prior Art

Recombinant DNA technology deals with techniques for manipulating, changing and transferring genes from one micro environment to another in order to produce useful proteins efficiently and inexpensively.

15 Depending on the biological system, a recombinant vector may be used directly to infect cells and cause production of foreign gene products both in tissue culture and live organisms.

Recombinant technology has been applied to
20 viruses including the insect baculovirus. When a baculovirus infects insect cells either in vitro or in vivo the virus produces large amounts of polyhedra, polymers of the 33kD polyhedrin protein. The polyhedrin encapsulates the virus particles in a
25 polyhedrin nucleocapsid and is the main agent for in vivo horizontal transmission of the virus, primarily, by preventing virus inactivation in the insect gut after ingestion. The polyhedrin protein is over-expressed in infected insect cells, and in
30 cultured insect cells can eventually account for up to 75% of the total cellular protein.

In European Patent No. 0,127,839 published December 12, 1984 it is reported that baculovirus DNA can be modified by recombinant methods to create a
35 useful vector by inserting a selected foreign gene or

portion thereof under the transcriptional control of a baculovirus promoter gene. The vector can then be used to allow a host insect cell to produce the selected protein.

5 In the work described in European Patent No. 0,127,839 the selected foreign gene was inserted in place of the gene which directed the high level production of the polyhedrin protein. Since the polyhedrin protein is not required for in vitro insect 10 cell infection, a foreign gene can be substituted for the gene for polyhedrin without affecting the in vitro viral replication of the recombinant virus. However, in the absence of the polyhedrin gene, the baculovirus has a much lower degree of infectivity in vivo if 15 ingested (L.E. Volkman et al., 19 J. Virol. 820 (1976)). Since the vector described by Smith et al. in European Patent No. 0,127,839 contains a foreign gene and does not contain the complete polyhedrin gene the resulting recombinant baculovirus (herein referred 20 to as the "recombinant polyhedrin negative baculovirus") has very low infectivity in insect larvae when ingested. This is due to inactivation of the unencapsulated virus in the gut of the insect.

Because of this drawback, the method of Smith 25 et al. is only practical for producing foreign proteins from infected insect tissue cultures. It was believed that it would be more efficient and desirable to have recombinant baculovirus express useful proteins in live insects rather than tissue culture.

30 The absence of the polyhedrin nucleocapsid however limited the usefulness of the recombinant viruses produced using the Smith et al. recombinant vectors in live insects.

The inability of the recombinant polyhedrin negative 35 baculovirus virus to infect live insects has been a serious limitation in the efforts to achieve efficient production of foreign protein in live insects. Some researchers have attempted to solve

this problem by microinjection of recombinant polyhedrin negative virus directly into live silk worm larvae (S. Maeda et al., Nature 315, 592 (1985)).

Although these scientists bypassed the problem of

5 digestion of the recombinant virus in the gut and achieved a reasonable level of expression of a foreign gene product in live silk worms, the microinjection is seen to be a practical and economic limitation to the feasibility of this method for commercial

10 applications.

Other attempts have been made to obtain expression of a foreign gene in a living animal but they have all suffered from the inability to incorporate easily the foreign gene into the animal

15 genome and obtain high levels of expression. One such approach has been the microinjection of foreign genes into embryonic cells of an animal (Jaenisch, R., and B. Mintz, 71 Proc. Natl. Acad. Sci. USA, 1250-1254 (1974)). Such models, however, have not given a high

20 level of expression of the foreign gene.

The current invention is a novel method which succeeds in allowing recombinant polyhedrin negative baculovirus to infect insects when ingested. It has now surprisingly been found that if an insect tissue culture is coinfectected with recombinant polyhedrin negative baculovirus and an appropriate amount of wild type baculovirus, the resulting product can then infect live insect colonies by ingestion and results in production of large quantities of the foreign

25 protein in live insects.

30

Summary of the Invention

The present invention is a method for making a recombinant polyhedrin negative baculovirus which contains a gene for a foreign protein, highly

35 infectious to live insects by ingestion and which results in the high level production of the foreign protein by the live insects.

Brief Description of the Drawings

Fig. 1 is a Cesium Chloride purification of insect hepatitis B virus surface antigen proteins (HBsAg) produced by the claimed process.

- 5 Fig. 2 is a graph illustrating the kinetics of HBsAg production by insect larvae in the claimed process.

Fig. 3 is an immunoblot of HBsAg protein produced by the claimed process.

10 Description of the Invention

It has now been found that recombinant baculovirus expression vectors under the transcriptional control of the polyhedrin promoter and having at least part of the polyhedrin gene deleted and replaced by a foreign gene can be adapted to produce high levels of the foreign protein in insects by coinfection of the recombinant baculovirus with the appropriate amount of wild-type baculovirus.

It is believed that the wild type baculovirus when used to coinfect insect tissue culture together with the recombinant polyhedrin negative virus synthesizes enough polyhedrin for itself as well as extra polyhedrin which can encapsulate the recombinant polyhedrin negative viruses in a nucleocapsid.

25 An insect cell culture, for example Spodoptera frugiperda coinjected with recombinant polyhedrin negative baculovirus as well as a wild type baculovirus produces polyhedrin-encapsulated particles which are believed to infect the insects by ingestion 30 or passive spraying and cause expression of the foreign genes in live insects. The ratio of recombinant polyhedrin negative virus to wild type baculovirus used in coinfection can vary but is generally from about 10:1 to 1:1.

35 The invention can be best understood by reference to specific examples which illustrate the method. The examples show the coinfection of cultures of

Spodoptera frugiperda with recombinant polyh drin negative Autographa californica incorporating the gene for hepatitis B virus surface antigen proteins (HBsAg), and wild type Autographa californica, to

- 5 produce a product which was used to infect live cabbage loopers (Trichoplusia ni). The method is equally applicable to other proteins which may be produced in tissue culture using recombinant baculovirus expression vectors, and to any of those
- 10 insects which are hosts for those expression vectors, such as species belonging to the order Lepidoptera and the order Hymenoptera. Suitable species for both coinfection and expression according to this process include but are not limited to Spodoptera frugiperda,
- 15 Bombyx mori and Trichoplusia ni.

Starting Materials

The following were used as starting material in the following examples:

- (a) Virus - Autographa californica Nuclear Polyhedrosis Virus (AcNPV) obtained from Dr. Max Summers, Texas A & M University and Texas Agricultural Experimental Station, College Station, Texas.
- 20 (b) Plasmid - pAc610 obtained from Dr. Max Summers.
- 25 (c) Foreign Gene - The gene coding for hepatitis B virus surface antigen of the ayw clone was produced in the laboratory of Dr. Peter Price, Mount Sinai School of Medicine of the City University of New York, New York, New York.
- 30 (d) Insect Cell Culture - Spodoptera frugiperda obtained from the American Type Culture Collection, Catalog No. CRL 1711.

In the examples standard recombinant DNA technological methods were used as described in T.

- 35 Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982.

Example 1Construction of Cloning Vector

The gene coding for hepatitis B virus surface antigen proteins (HBsAg) was excised from the ayw 5 clone of hepatitis B virus DNA and inserted into the plasmid pAc610 by standard recombinant techniques employing restriction endonucleases and ligases.

Example 2Transfection to Create Recombinant Baculovirus

10 The chimeric plasmid thus created, was cotransfected into tissue culture along with wild type baculovirus (Autographa californica Nuclear Polyhedrosis Virus [AcNPV]) DNA to facilitate random recombination events. From this culture, dilution 15 plating was used to select recombinants containing the DNA of HBsAg integrated into the viral genome in place of the viral polyhedrin gene. In these recombinants, the promoter of the polyhedrin gene was left intact, and so was able to regulate transcription of the 20 substituted HBsAg gene.

The recombinant virus had been constructed to contain the gene for the HBsAg in place of the polyhedrin gene such that the HBsAg gene is controlled by the polyhedrin promoter (V.A. Luckow and M.D. 25 Summers, 6 Biotechnology 47 (1988); P.M. Price et al. 7 DNA 417 (1988)).

Example 3Coinfection to Encapsulate Recombinant Polyhedrin Negative Baculovirus

30 Cultured cells of Spodoptera frugiperda were coinjected with wild-type baculovirus (Autographa californica Nuclear Polyhedrosis Virus (AcNPV)) and the recombinant polyhedrin negative virus containing the HBsAg DNA from Example 2 in either 10:1, 1:1, or 35 1:10 ratios of recombinant to wild baculovirus. After five days in culture, the medium containing virus from

each of the combinations was separately collected using a pasteur pipette.

Example 4

Infection of Insect Larvae

5 The media from Example 3 was fed to larvae of the cabbage looper, Trichoplusia ni in their drinking water. The larvae had been reared on semi-synthetic diet and kept at 28°C and 60% humidity. After molting to become fourth instar larvae, the insects were
10 removed from the diet and starved for seven hours. The insects were placed in contact with the insect cell culture medium containing a dosage of media from Example 3 equivalent to the LD₉₅ of the wild-type virus (P.R. Hughes and H.A. Wood, 37 J. Invent. PATH
15 154 (1981)). After one hour, the larvae were placed onto fresh semi-synthetic diet and kept as before. The larvae exhibited successful infection, and as a result their cells produced normal viral proteins as well as a significant amount of hepatitis B surface
20 antigen protein (HBsAg).

Example 5

Harvesting Larvae

At various times post-infection (1 to 5 days), moribund larvae were collected and frozen at -70°C
25 until HBsAg determinations could be performed. For the determination of the HBsAg concentration, approximately 100 mg of insect (wet weight) was added to ten volumes of NME (150 mM NaCl, 25 mM MES, pH 7.0, 1 mM EDTA, 0.1 mM 2-mercaptoethanol, 0.02% NaN₃) on
30 ice and sonicated for 45 seconds. Triton-X-100 was added to 0.5% and the suspension centrifuged at 16,000 X G for 20 minutes. The lipid layer of the supernatant was removed by aspiration and the rest of the supernatant was then collected.

Example 6Protein and HBsAg Assessment

Protein and HBsAg concentrations were performed by a BioRad protein determination kit and a solid 5 phase RIA (Connaught), respectively. The HBsAg detected by this latter assay is a conformational antigen.

The supernatant from an insect larval lysate prepared as described above was diluted five-fold with 10 NME buffer and adjusted to a density of 1.2 g/ml by addition of 230 mg CsCl per ml. The sample was centrifuged in a Ti 70.1 rotor at 46,000 rpm for 42 hours at 5°C. Fractions were collected, and those containing maximum HBsAg activity as shown in Fig. 1 15 (shaded area) were pooled and dialyzed against NME buffer.

The yield of HBsAg was approximately 0.15 mg per insect, representing 1.5% of the total extracted protein (Kinetics shown in Fig. 2). This high protein 20 yield in the live insect system is consistent with other reports indicating that although the expression of the polyhedrin promoter is very high in cultured cells, the yield of polyhedrin is significantly more economical in live insect larvae (T.R. Shieh and G.T. 25 Bohmfalk, 22 Biotech. & Bioeng. 1357, (1980)).

The density (1.2 gm per ml, Fig. 1) and morphologic appearance as determined by electro-microscopy of the recombinant HBsAg were identical to that of HBsAg isolated from the serum of infected 30 patients.

Example 7Immunoblot Analysis

Aliquots from dialyzed HBsAg (Fig. 1) were electrophoresed on 15% PAGE (U.K. Laemmli, 227 Nature 680 35 (1970)) and either stained for protein (BioRad Silver Stain) or transferred to nylon (H. Towbin et al., 162 Anal. Biochem. 389 (1987)). The nylon was blocked for

16 hours with 5% nonfat dry milk, TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20), 0.1% NaN₃, 2 µg per ml bovine IgG. The primary antibody (monoclonal anti HBsAg, a gift from Dr. L. Mimms, Abbott Labs) was
5 diluted into TBST and incubated with the nylon for 16 hours. After washing, the antibody was detected with an alkaline phosphatase-coupled secondary antibody (Promega Biotec). Lanes 1 and 2 were silver stained; lane 3 was immunoblotted (Fig. 3). Lane 1, molecular
10 weight markers. Lanes 2 and 3 are 750 ng of HBsAg prepared as described above. Major species of proteins which are immunoreactive with anti-HBsAg are present at 24 and 27 kD. Another immunoreactive protein at 21 kD is most likely the result of specific
15 protease cleavage, as has been noted to occur in HBV envelope proteins (W. Stibbe and W. Gerlich, 46 J. Virol. 426 (1983); Y. Itoh et al., 138 Biochem. Biophys. Res. Comm. 268 (1986)).

The results demonstrated the presence of both
20 unglycosylated (24 kD) and glycosylated (27 kD) forms of HBsAg. This is a similar profile to that produced by infected insect cells in vitro, although larger molecular weight forms of HBsAg formed by initiation of translation at upstream sites in infected cultured
25 cells (P.M. Price et al., 7 DNA 417 (1988)) were not detected. Also, these molecular weight species are the same as those of HBsAg isolated from the serum of HBV carriers (K.H. Heerman, et al., 52 J. Virol. 396 (1984)).

30

Example 8Immunogenicity Determination

Ten-week-old female BALB/c mice were injected via footpad, with 10 µg HBsAg isolated from the peak fraction of a cesium chlorid gradient (Fig. 1), both
35 with and without complete Freund's adjuvant (CFA). The serum was tested two weeks later for anti-HBsAg antibodies. The procedure of Khan, et al. was adapted

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for measuring ELISA anti-HBsAg titers (M.W. Khan, et al., 16 J. Clin. Microbiol. 115 (1982)). Microtiter plate wells were coated with 40 ng per well using HBsAg prior to applying the mouse serum. The
5 mouse serum was diluted from 1:300 up to 1:24,300 using PBS-Tween (0.15 M phosphate-buffered saline, pH 7.4, 0.05% Tween 20) containing 2% tissue culture medium from wild-type baculovirus infected cells. The binding of mouse antibody to the HBsAg was determined
10 using alkaline phosphatase-conjugated sheep anti-mouse immunoglobulin (Sigma).

Two weeks after injection, the ELISA anti-HBsAg titer of five mice injected without CFA ranged from 372 to 626, and the titer of five mice injected with
15 CFA ranged from 9,053 to 20,412. These results demonstrate that the HBsAg produced in large quantity by the live insect larvae is indeed immunogenic.

Claims

1. A method for producing high levels of foreign protein by the expression of foreign genes in live insects comprising:
 - 5 (a) coinfecting in a medium an insect cell culture with a recombinant polyhedrin negative baculovirus and wild type baculovirus in ratios of from 10:1 to 1:1 and 1:1 to 1:10, respectively;
 - 10 (b) administering the medium containing the recombinant virus from the coinfection to insects;
 - (c) allowing the recombinant virus to replicate in the insects;
 - 15 (d) harvesting and lysing the insects;
 - (e) purifying the lysate.
2. The method of claim 1, wherein the foreign protein is hepatitis B virus surface antigen (HBsAg) proteins, or a part thereof.
- 20 3. The method of claim 2, wherein the foreign protein is hepatitis B virus surface antigen (HBsAg) of the ayw strain of the hepatitis B virus.
4. The method of claim 1, wherein the wild type baculovirus is Autographa californica.
- 25 5. The method of claim 1, wherein the recombinant polyhedrin negative baculovirus genome comprises at least one foreign gene or part thereof, said foreign gene or part thereof being under the transcriptional regulation of a baculovirus promoter.

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6. The method of claim 5, wherein said baculovirus promoter is the polyhedrin promoter.
7. The method of claim 1, wherein the foreign gene in the recombinant polyhedrin negative baculovirus has substituted itself for all or part of a baculovirus gene.
5
8. The method of claim 1, wherein the insect cells which are coinfecte^d are selected from the group consisting of Spodoptera frugiperda, Bombyx mori, and Trichoplusia ni.
10
9. The method of claim 1, wherein the ratios of recombinant to wild-type baculovirus used in coinfection were: 10:1, 1:1, or 1:10.
10. The method of claim 9, wherein the ratio of recombinant to wild-type Autographa californica is 10:1.
15
11. The method of claim 1, wherein the medium containing the recombinant virus is administered to the insect by ingestion.
- 20 12. The method of claim 1, wherein the insects administered the medium are selected from the group consisting of species belonging to the order Lepidoptera and the order Hymenoptera.
13. The method of claim 1, wherein the insects administered the medium are selected from the group consisting of Spodoptera frugiperda, Bombyx mori and Trichoplusia ni.
25
14. The methods of claim 1, wherein the insects administered the medium are those of Trichoplusia ni.
30

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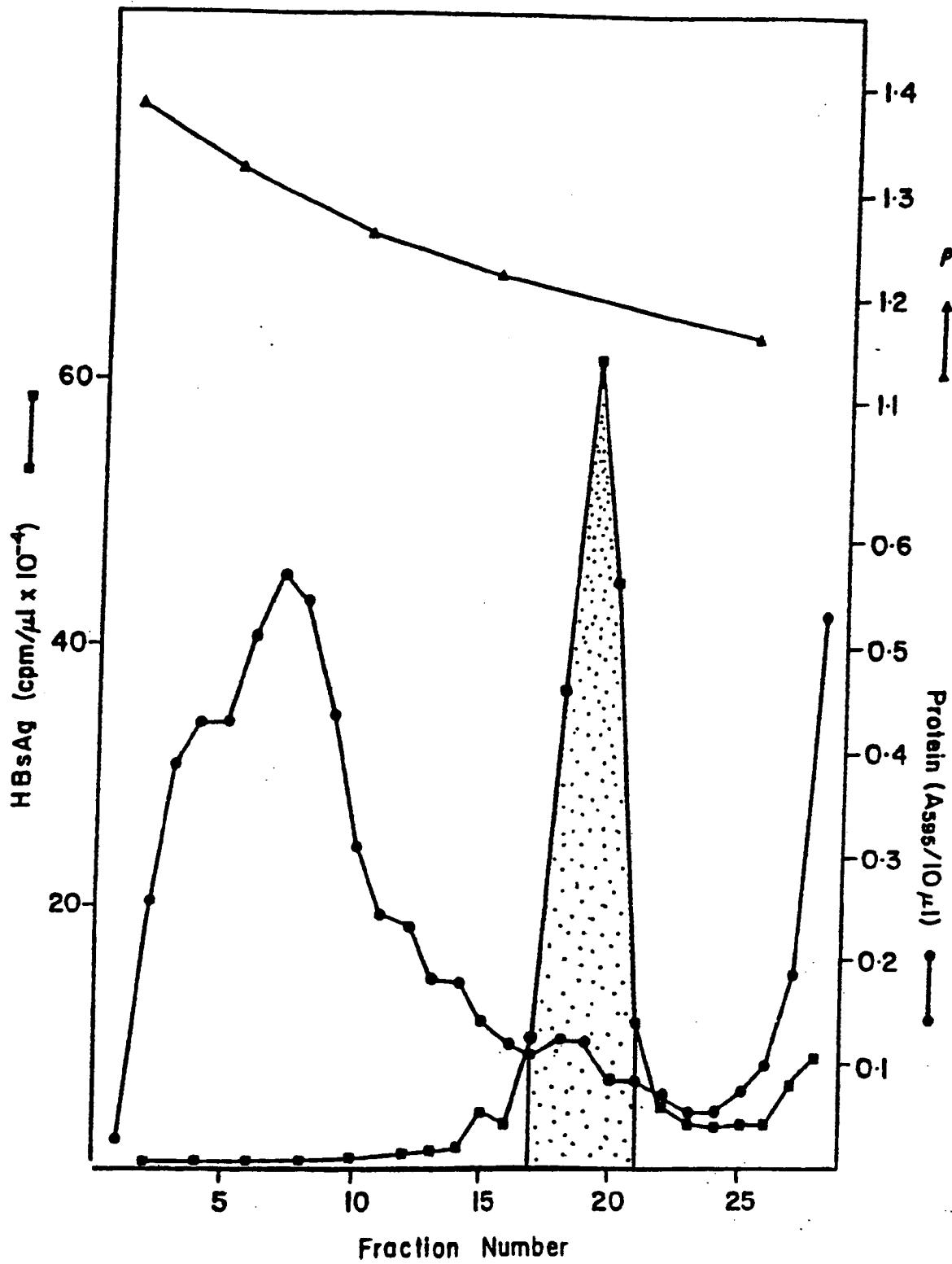


FIG. I
SUBSTITUTE SHEET

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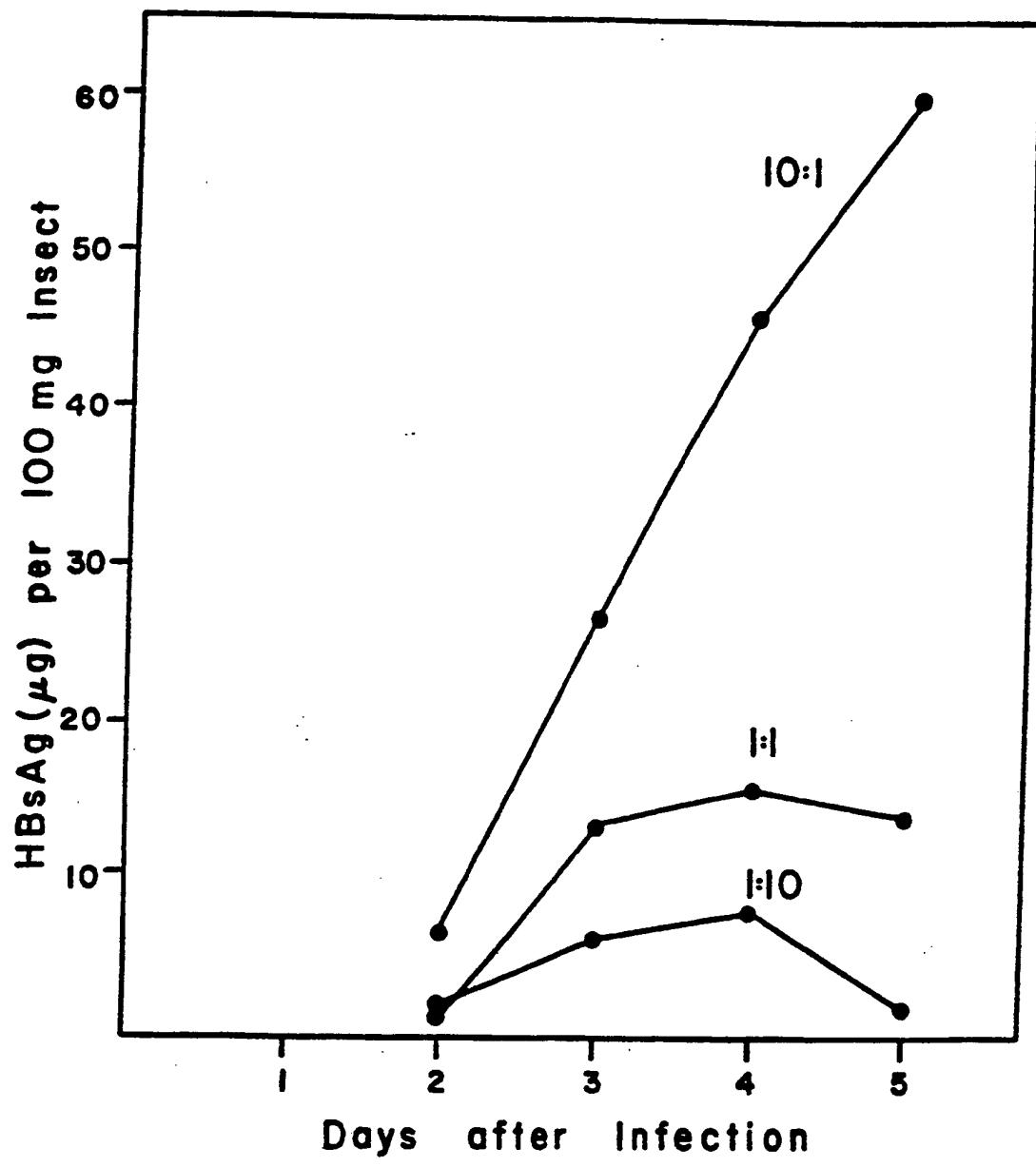


FIG. 2

SUBSTITUTE SHEET

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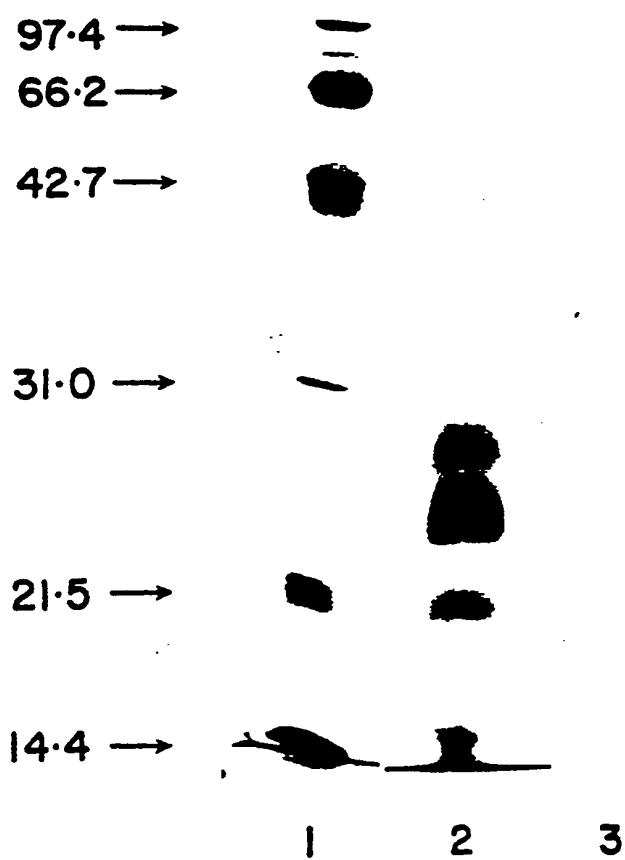


FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 89/03542

I. CLASSIFICATION & SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

⁵ IPC : C 12 P 21/02, C 12 N 15/86, C 12 N 15/36

II. FIELDS SEARCHED

Minimum Documentation Searched ?

Classification System	Classification Symbols
IPC	C 12 N, C 12 P

Documentation Searched other than Minimum Documentation
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III. DOCUMENTS CONSIDERED TO BE RELEVANT*

Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO, A, 88/02030 (GENETICS INSTITUTE INC.) 24 March 1988, see the whole document --	1-14
A	Biotechnology, vol. 6, no. 5, January 1988, (New York, US), V.A. Luckow et al.: "Trends in the development of baculovirus expression vectors", pages 47-55, see table 1 --	2,3
A	EP, A, 0222412 (DAIICHI SEIYAKU CO. LTD) 20 May 1987, see page 8, line 5 --	11
P,X	Proceedings of the National Academy of Sciences of the USA, vol. 86, no. 5, March 1989 (Washington, D.C., US), P.M. Price et al.: "Complementation of recombinant baculoviruses by coin- fection with wild-type virus facilitates production in insect larvae of antigenic proteins of hepatitis B virus and influ- enza virus", see pages 1453-1456 ----	1-14

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IV. CERTIFICATION

Date of the Actual Completion of the International Search

15th November 1989

Date of Mailing of this International Search Report

05.12.89

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

F.M. VRIJDAG

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. US 8903542
SA 30869**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 22/11/89. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A- 8802030	24-03-88	AU-A-	8032387	07-04-88
		EP-A-	0323974	19-07-89
EP-A- 0222412	20-05-87	JP-A-	62208276	12-09-87